

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference H0535/7011WO	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US 00/20211	International filing date (day/month/year) 24/07/2000	(Earliest) Priority Date (day/month/year) 22/07/1999
Applicant PERICOR SCIENCE, INC.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

- contained in the international application in written form.
- filed together with the international application in computer readable form.
- furnished subsequently to this Authority in written form.
- furnished subsequently to this Authority in computer readable form.
- the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. Certain claims were found unsearchable (See Box I).

3. Unity of Invention is lacking (see Box II).

4. With regard to the title,

- the text is approved as submitted by the applicant.
- the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- the text is approved as submitted by the applicant.
- the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

- as suggested by the applicant.
- because the applicant failed to suggest a figure.
- because this figure better characterizes the invention.

None of the figures.



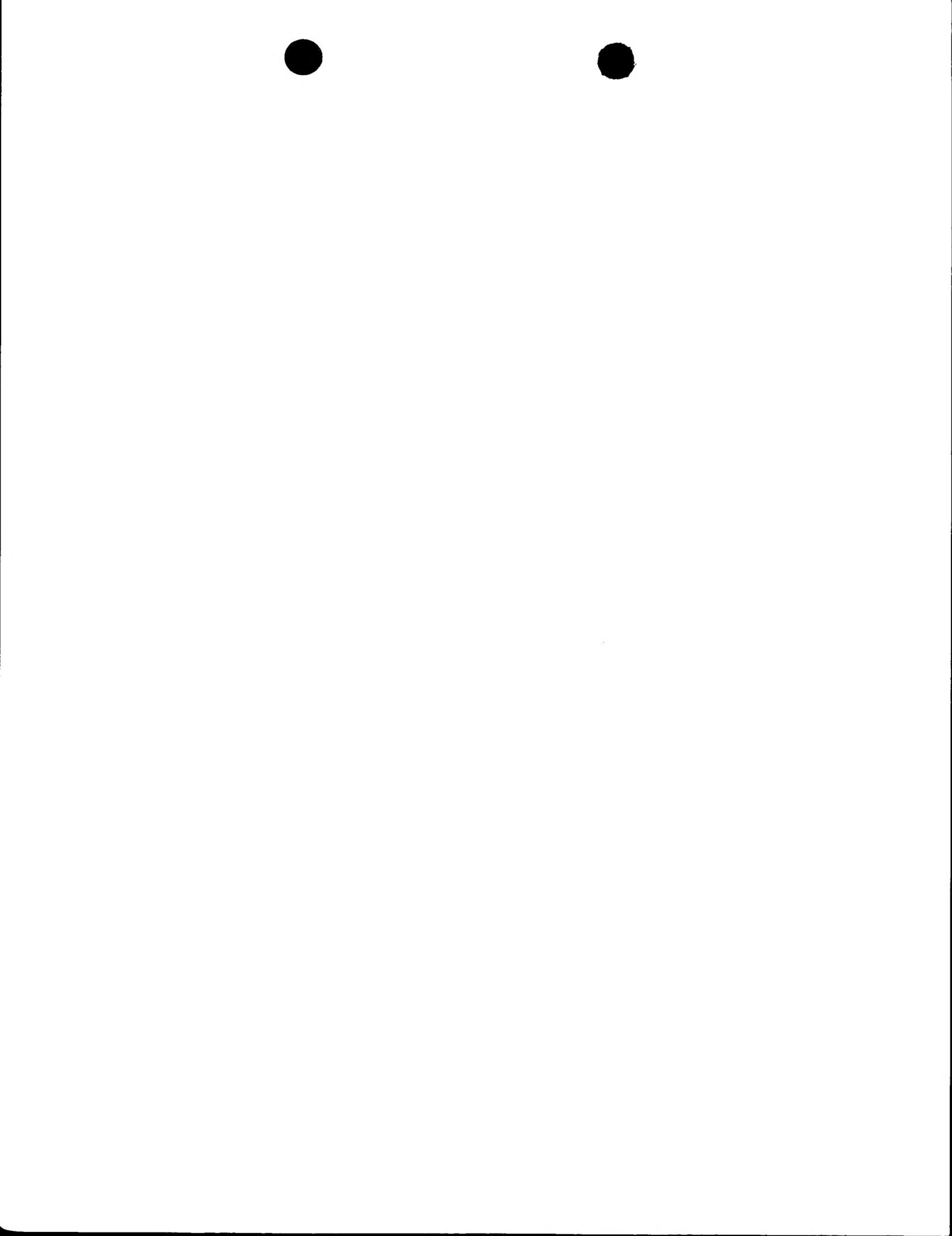
FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: Claims 1-90 (all partial)

Present claims 1-90 relate to an extremely large number of possible methods, kits and compositions. In fact, the claims contain so many options that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. In particular the terms "linker" and "agent" used in claim 1 and thereafter in the 89 following submitted claims, insofar as these terms relate to the subject matter of any independent claim, refer to vague compounds. Consequently, the search has been carried out for those parts of the application which do appear to be clear, namely : linkers have been restricted to compounds cited in claims 3-5,10-11 and 26; agents have been restricted to those appearing in claims 16-19, 32, 46, 69 and 85.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/20211

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K7/48 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 41215 A (NOVO NORDISK) 6 November 1997 (1997-11-06) cited in the application the whole document ---	1
A	US 5 490 980 A (N. RICHARDSON ET AL.) 13 February 1996 (1996-02-13) cited in the application the whole document ---	1
A, P	WO 99 36570 A (PERICOR SCIENCE, INC.) 22 July 1999 (1999-07-22) the whole document ---	1
A	EP 0 615 745 A (H. GREEN ET AL.) 21 September 1994 (1994-09-21) the whole document ---	1
	-/-	

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

7 November 2000

Date of mailing of the international search report

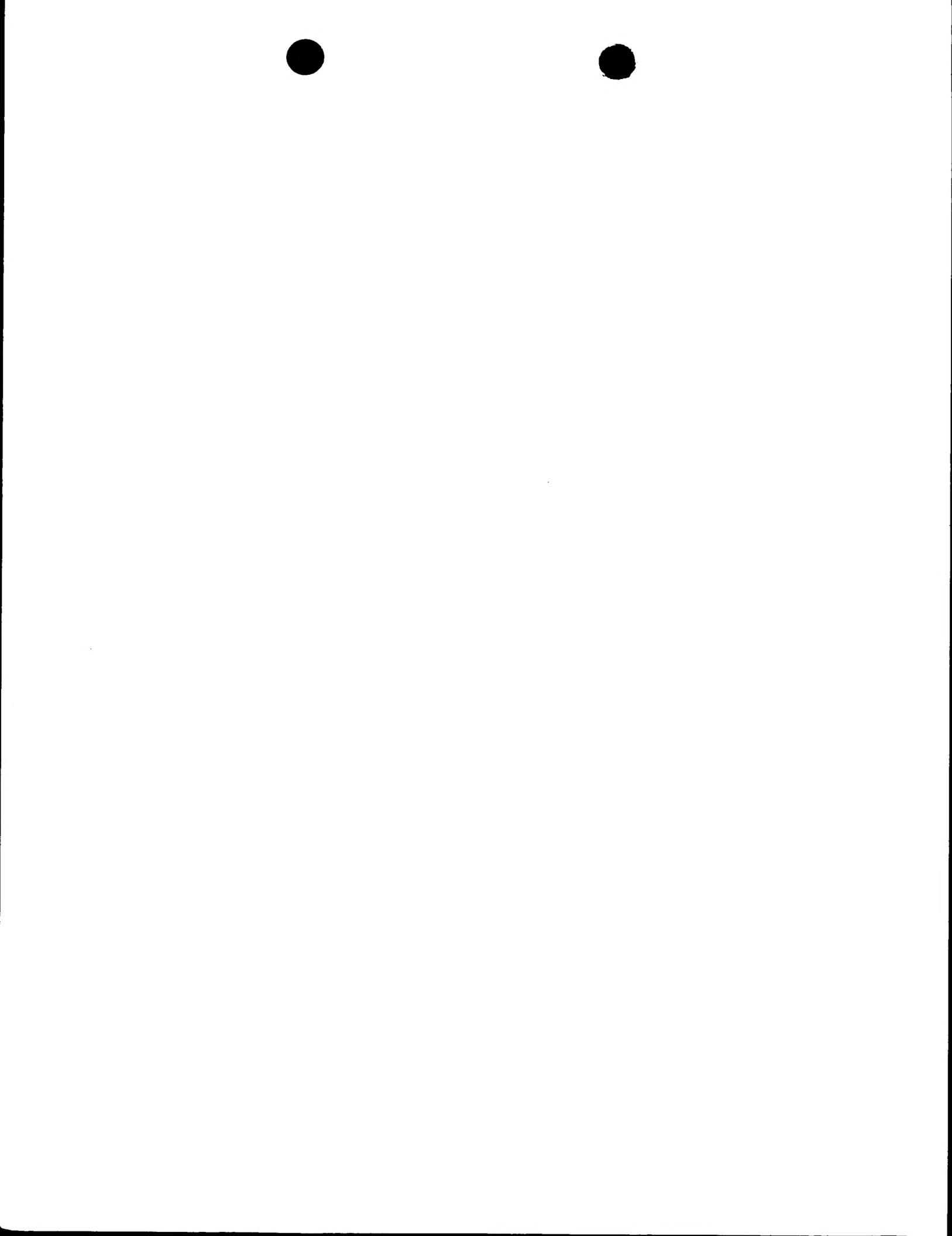
15/11/2000

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 European Patent Office, P.B. 5818 Patentaan 2
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 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Glikman, J-F

1



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/20211

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 773 577 A (J. CAPPELLO) 30 June 1998 (1998-06-30) column 3, line 15 -column 4, line 31 -----	1



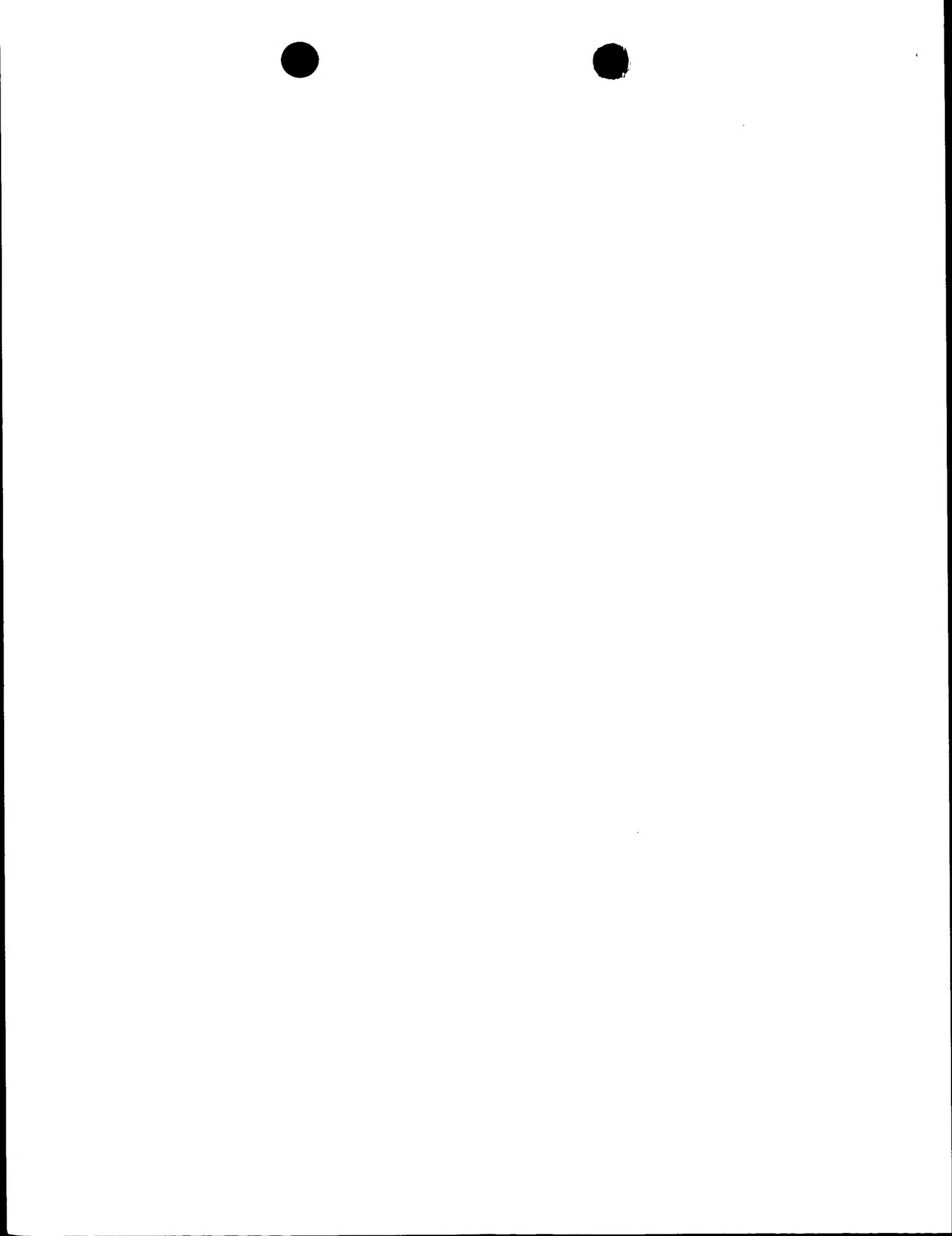
INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/20211

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9741215	A	06-11-1997	AU 2635097 A		19-11-1997
			EP 0896618 A		17-02-1999
US 5490980	A	13-02-1996	NONE		
WO 9936570	A	22-07-1999	NONE		
EP 615745	A	21-09-1994	AU 694104 B		16-07-1998
			AU 6083994 A		14-09-1994
			CA 2155408 A		01-09-1994
			DE 69310339 D		05-06-1997
			DE 69310339 T		21-08-1997
			ES 2101263 T		01-07-1997
			JP 8506830 T		23-07-1996
			WO 9418945 A		01-09-1994
			US 5525336 A		11-06-1996
US 5773577	A	30-06-1998	AU 694196 B		16-07-1998
			AU 1979295 A		18-09-1995
			CA 2183643 A		08-09-1995
			EP 0752878 A		15-01-1997
			JP 9509840 T		07-10-1997
			WO 9523611 A		08-09-1995



INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00194

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/96, C11D 3/386 // C12N 9/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4943530 A (JUERGEN CHRISTNER ET AL), 24 July 1990 (24.07.90), column 5, line 68 - column 6, line 8; column 12, line 39 - line 45 --	1-14
A	WO 9412620 A1 (NOVO NORDISK A/S), 9 June 1994 (09.06.94), the whole document -- -----	1-14

 Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

30 July 1997

Date of mailing of the international search report

04-08-1997

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Swedish Patent Office
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Facsimile No. +46 8 666 02 86

Authorized officer

Patrick Andersson
Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
01/07/97 PCT/DK 97/00194

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4943530 A	24/07/90	AU 618523 B AU 1165688 A CH 677798 A DE 3704465 A,C FI 93656 B,C FR 2610947 A GB 2201960 A,B JP 63216480 A KR 9616075 B SE 500913 C SE 8800238 A US 5102422 A WO 8806183 A	02/01/92 18/08/88 28/06/91 25/08/88 31/01/95 19/08/88 14/09/88 08/09/88 27/11/96 26/09/94 14/08/88 07/04/92 25/08/88
WO 9412620 A1	09/06/94	CA 2150562 A CA 2150563 A EP 0677102 A EP 0679183 A FI 952647 A FI 952648 A JP 8503371 T JP 8506009 T WO 9412621 A	09/06/94 09/06/94 18/10/95 02/11/95 31/05/95 28/07/95 16/04/96 02/07/96 09/06/94

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number
WO 01/07009 A1

(51) International Patent Classification⁷: A61K 7/48, 47/48

(74) Agent: GATES, Edward, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

(21) International Application Number: PCT/US00/20211

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(22) International Filing Date: 24 July 2000 (24.07.2000)

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English

(26) Publication Language: English

Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(30) Priority Data:

09/359,987 22 July 1999 (22.07.1999) US

(71) Applicant (*for all designated States except US*): PERICOR SCIENCE, INC. [US/US]; One Kendall Square, Building 600, PMB 299, Cambridge, MA 02139 (US).

(72) Inventors; and

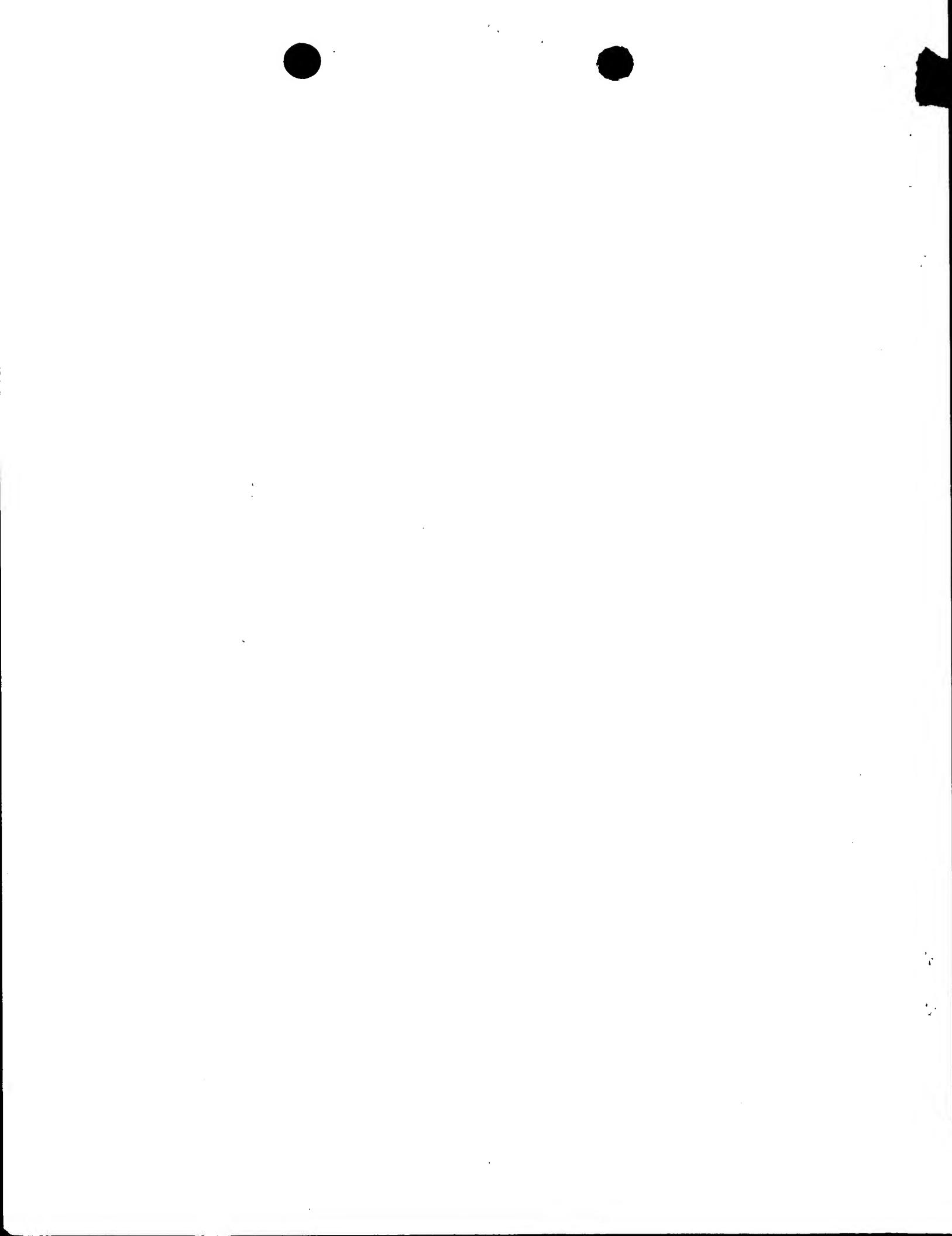
(75) Inventors/Applicants (*for US only*): GREEN, Howard [US/US]; 82 Williston Street, Brookline, MA 02146 (US). RANDO, Robert, R. [US/US]; 60 Montvale Road, Newton Center, MA 02459 (US).



WO 01/07009 A1

(54) Title: LYSINE OXIDASE LINKAGE OF AGENTS TO TISSUE

(57) Abstract: Methods, products and kits are provided for attaching agents to body tissues using lysine oxidase.



PATENT COOPERATION TREATY

JED

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From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
EDWARD R. GATES
WOLF, GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210

PCTNOTIFICATION OF TRANSMITTAL OF
INTERNATIONAL PRELIMINARY
EXAMINATION REPORT

(PCT Rule 71.1)

		Date of Mailing (day/month/year) 07 NOV 2001
Applicant's or agent's file reference H0535/7011WO		IMPORTANT NOTIFICATION
International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/US00/20211	24 July 2000 (24.07.2000)	22 July 1999 (22.07.1999)
Applicant PERICOR SCIENCE, INC.		

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

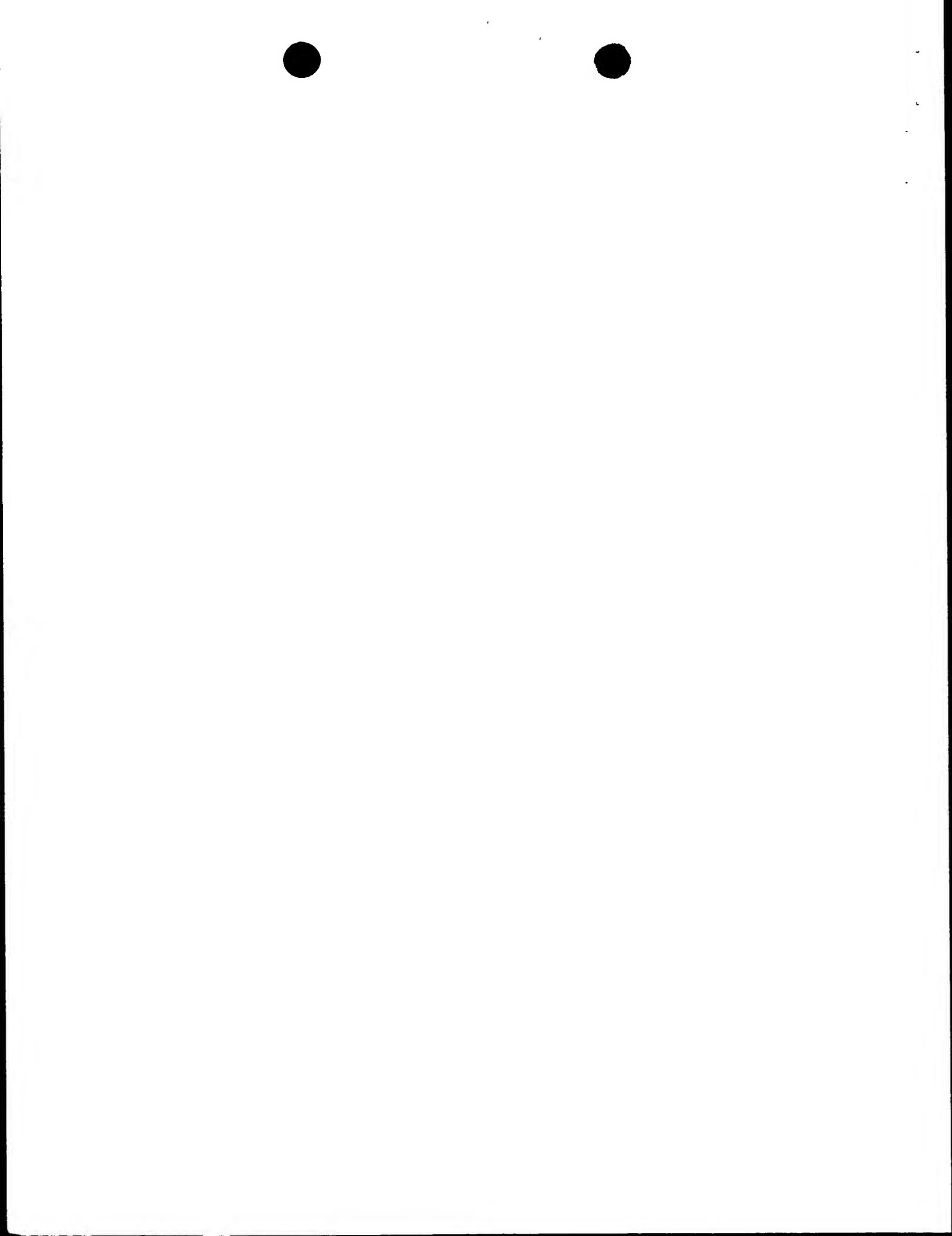
For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Jean C. Witz Telephone No. (703) 308-0196
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Form PCT/IPEA/416 (July 1992)

File Folder	Initials
ECB	<input checked="" type="checkbox"/>
Docket Entry	<input type="checkbox"/>
Docket Cross Off	<input checked="" type="checkbox"/> b2
Order Copies	<input type="checkbox"/>
Annuities	<input type="checkbox"/>
Confirmation	<input type="checkbox"/>

DOCKETED
NOV 26 2001



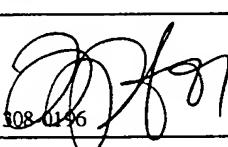
INTERNATIONAL COOPERATION TREATY
PCT
 INTERNATIONAL PRELIMINARY EXAMINATION REPORT
 (PCT Article 36 and Rule 70)

14

REC'D 12 NOV 2001
USPTO FEB

Applicant's or agent's file reference H0535/7011WO	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/20211	International filing date (day/month/year) 24 July 2000 (24.07.2000)	Priority date (day/month/year) 22 July 1999 (22.07.1999)
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 7/48, 47/48 and US Cl.: 424/94.63		
Applicant PERICOR SCIENCE, INC.		

<ol style="list-style-type: none"> 1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36. 2. This REPORT consists of a total of <u>3</u> sheets, including this cover sheet. <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of ___ sheets.</p>
<ol style="list-style-type: none"> 3. This report contains indications relating to the following items: <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input type="checkbox"/> Non-establishment of report with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input type="checkbox"/> Certain observations on the international application

Date of submission of the demand 09 February 2001 (09.02.2001)	Date of completion of this report 01 July 2001 (01.07.2001)
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Jean C. Witz Telephone No. (703) 308-0146 

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International Application No.

PCT/US00/20211

I. Basis of the report

1. With regard to the elements of the international application:*

the international application as originally filed.

the description:
pages 1-82 _____ as originally filed
pages NONE _____, filed with the demand
pages NONE _____, filed with the letter of _____

the claims:
pages 83-95 _____, as originally filed
pages NONE _____, as amended (together with any statement) under Article 19
pages NONE _____, filed with the demand
pages NONE _____, filed with the letter of _____

the drawings:
pages 1 _____, as originally filed
pages NONE _____, filed with the demand
pages NONE _____, filed with the letter of _____

the sequence listing part of the description:
pages NONE _____, as originally filed
pages NONE _____, filed with the demand
pages NONE _____, filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

the language of publication of the international application (under Rule 48.3(b)).

the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

contained in the international application in printed form.

filed together with the international application in computer readable form.

furnished subsequently to this Authority in written form.

furnished subsequently to this Authority in computer readable form.

The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

the description, pages NONE

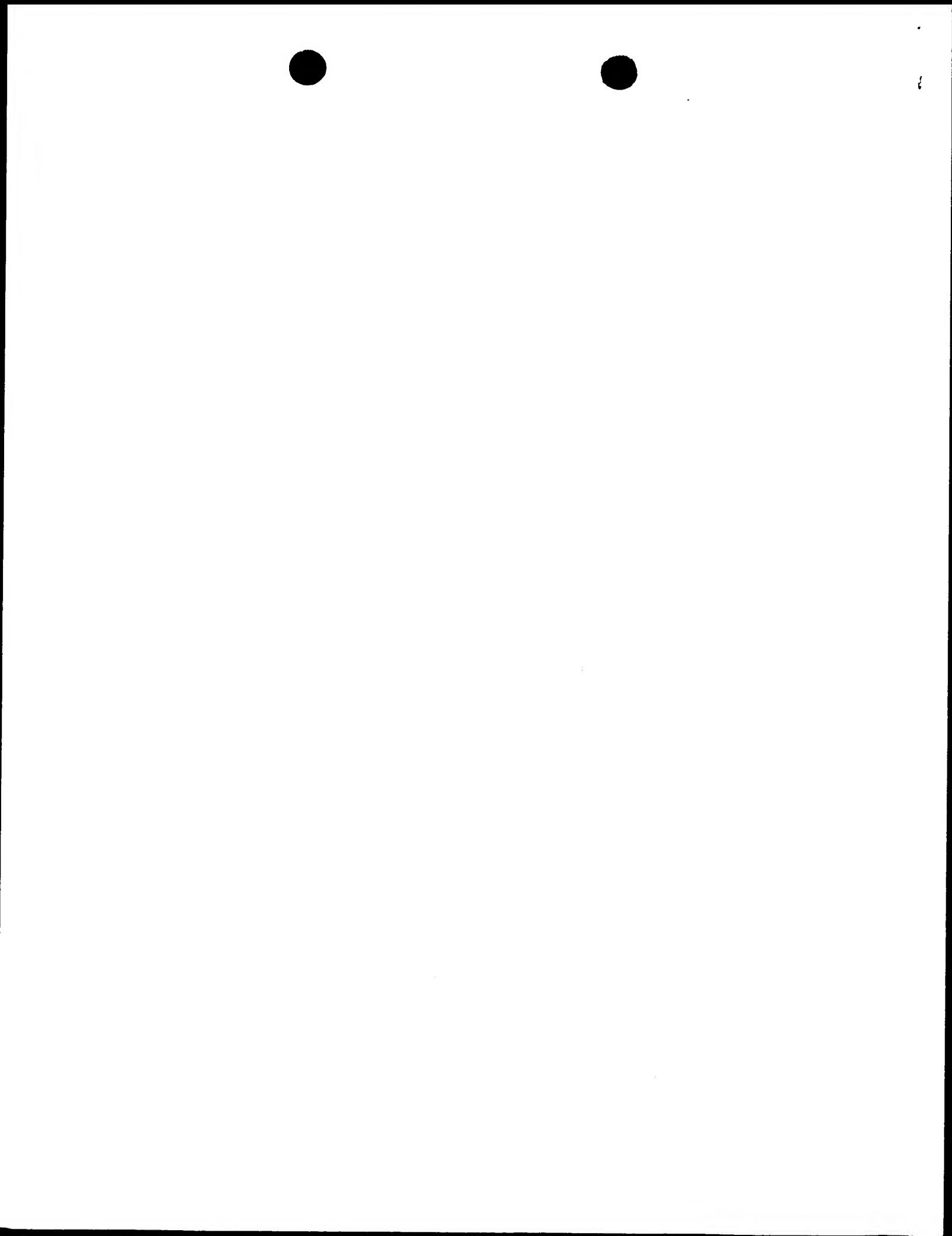
the claims, Nos. NONE

the drawings, sheets/fig NONE

5. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International Application No.

PCT/US00/20211

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N) Claims 1-90 YES
 Claims NONE NO

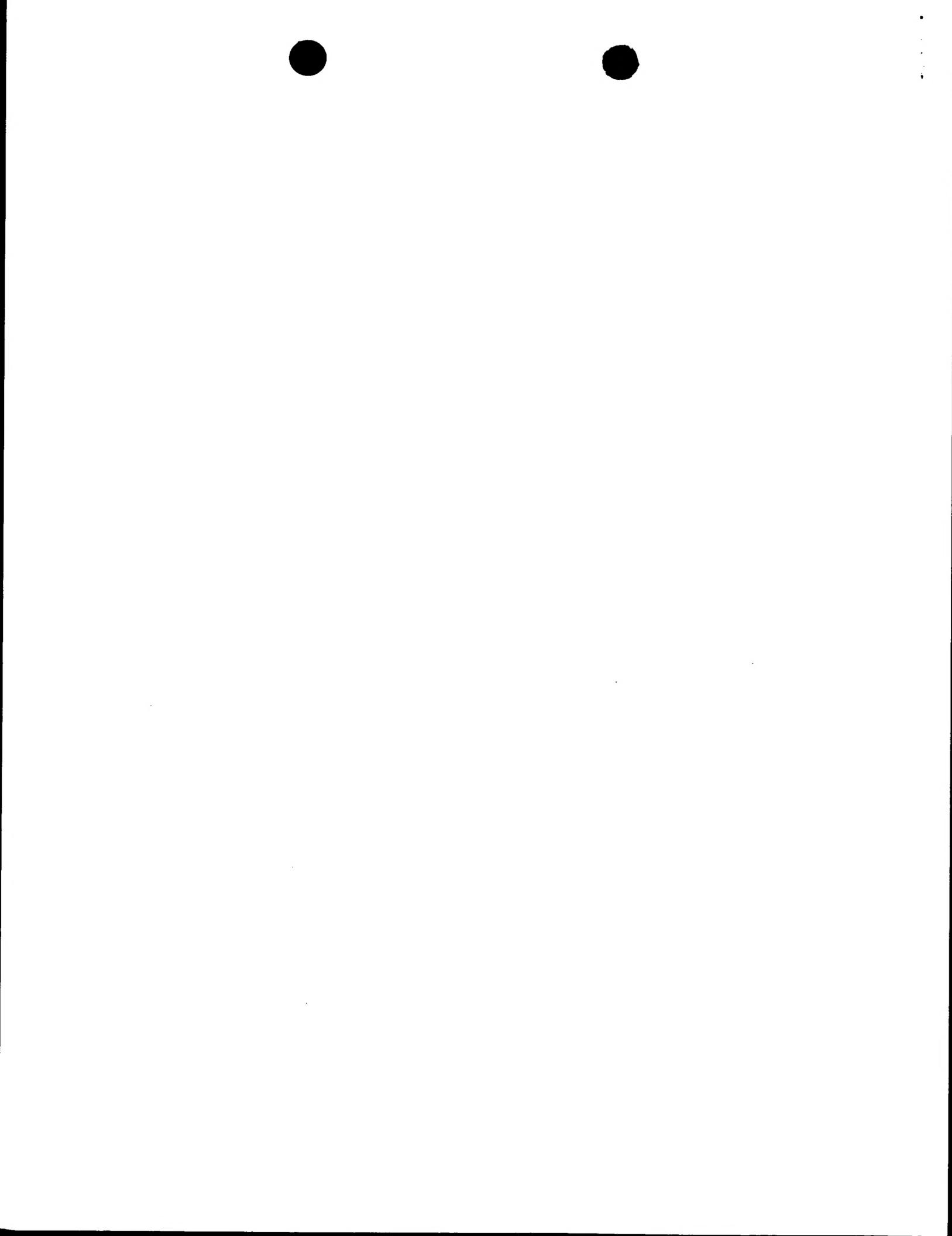
Inventive Step (IS) Claims 1-90 YES
 Claims NONE NO

Industrial Applicability (IA) Claims 1-90 YES
 Claims NONE NO

2. CITATIONS AND EXPLANATIONS (Rule 70.7)

Claims 1-90 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest the use of lysine oxidase for attaching an agent to a body tissue.

----- NEW CITATIONS -----
NONE





Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number:

0 615 745 A1

(12)

EUROPEAN PATENT APPLICATION

(2) Application number: **93402240.1**

(51) Int. Cl.5: **A61K 7/48, C07K 15/06**

(22) Date of filing: **15.09.93**

(30) Priority: **19.02.93 US 20212**

(43) Date of publication of application:
21.09.94 Bulletin 94/38

(84) Designated Contracting States:
**AT BE CH DE DK ES FR GB GR IE IT LI LU MC
NL PT SE**

(71) Applicant: **Green, Howard
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Applicant: **Djian, Philippe
18, rue Ferdinand Fabre
F-75015 Paris (FR)**

(72) Inventor: **Green, Howard
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Brookline, Massachusetts 02146 (US)**
Inventor: **Djian, Philippe
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F-75015 Paris (FR)**

(74) Representative: **Casalonga, Axel
BUREAU D.A. CASALONGA - JOSSE
Morassistrasse 8
D-80469 München (DE)**

(52) Compositions containing corneocyte proteins.

(57) Cosmetic compositions containing corneocyte proteins or polypeptides in a cosmetically acceptable vehicle can be used to form a protective layer on skin, hair or nails. The proteins may be crosslinked, thereby forming a protective layer which mimicks the corneal layer of natural skin.

EP 0 615 745 A1

Background of the Invention

The epidermis is a stratified epithelium consisting of numerous cell layers. The principal cell type of the epidermis is the keratinocyte. Keratinocytes are responsible for the resistance of the skin to physical and chemical injury and for its impermeability to water. These functions are carried out primarily by the outermost layer of keratinocytes, termed "corneocytes," which have undergone programmed cell death. Corneocytes consist of a skeletal framework of keratin filaments surrounded by a special envelope that is found in no other cells within the body. This envelope consists of proteins ("corneocyte proteins") stabilized by inter-molecular isopeptide bonds (Rice and Green (1977), *Cell*, 11:417-422). The bonds are introduced by an enzyme, calcium-activated keratinocyte transglutaminase (Rice and Green (1979), *Cell*, 18:681-694).

As skin ages, the epidermis atrophies with accompanying diminution of its physical resistance.

The basal layer of the epidermis contains proliferating keratinocytes. When keratinocytes leave the basal layer, they begin to undergo terminal differentiation. When they reach the granular layer, the concentration of calcium ions inside the keratinocytes rises, resulting in activation of transglutaminase. The envelope precursor proteins, which at this stage are located just beneath the plasma membrane, are crosslinked by transglutaminase (Rice and Green (1979), *ibid.*; Greenberg et al. (1991) *FASEB*, 5:3071-3077) and the resulting envelope becomes thoroughly insoluble, even in the presence of detergents and reducing agents (Green (1977), *Cell*, 11:405-416; Hohl (1990), *Dermatologica*, 180:201-211). This envelope is the most resistant structure of the skin since keratin filaments, even though they are stabilized by disulfide bonds, can be dissolved by a combination of detergent and reducing agent. Transglutaminase and its crosslinked products are also present in hair and nails (Rice et al., *Keratinocyte Handbook*, (in press)).

Summary of the Invention

The invention comprises a composition suitable for topical application to mammalian skin, hair and/or nails comprising a corneocyte protein, or polypeptide fragment thereof. The proteins or polypeptides are dispersed in a cosmetically acceptable vehicle which is designed to permit effective application of the proteins or polypeptides to the skin, hair or nails. A method for forming a protective layer on mammalian skin, hair or nails by applying a topical cosmetic composition containing an effective amount of the proteins or polypeptides also is the subject of the present invention.

The composition alternatively may comprise a crosslinking agent which is specific for corneocyte proteins, i.e., which forms crosslinks or induces crosslinking between corneocyte proteins already present in the stratum corneum. The crosslinking agent may be a chemical agent which reacts with two or more functional groups present in amino acid side chains of the proteins thereby forming crosslinks between the amino acids of different proteins. Chemical crosslinkers, such as glutaraldehyde, typically are themselves incorporated into the crosslink. Alternatively, the crosslinking agent may be an enzyme, such as transglutaminase, which induces formation of crosslinks between glutamine and lysine residues of adjacent polypeptides.

Corneocyte proteins which are preferred for use in the present composition and method include involucrin, loricrin, cornifin, trichohyalin, scieillin, profilaggrin and keratolinin and/or polypeptide fragments of these proteins. The proteins or polypeptides may be natural-sourced or recombinant. The proteins or polypeptides may be crosslinked, if desired, using a chemical crosslinker or an enzyme which forms bonds between adjacent polypeptides. Optionally, the proteins or polypeptides may be modified, for example, by chemically derivatizing or substituting one or more of the amino acids in the protein or polypeptide chain. Such modifications can be designed to impart specific properties to the protein or polypeptide for a selected application.

In a preferred embodiment, a protective layer is formed on skin, hair or nails by applying thereto a composition containing one or more corneocyte proteins or polypeptides. The proteins or polypeptides may be crosslinked to form the protective layer. For this purpose, a crosslinking agent capable of inducing formation of isopeptide bonds between molecules of the protein or polypeptide preferably is included in the composition. In another embodiment, a composition comprising only the crosslinking agent is applied, thereby inducing crosslinking of proteins already present in the skin, hair or nails. The presence of the crosslinking agent induces formation of a crosslinked protein network analogous to the corneocyte envelope which occurs naturally in skin. The protein network forms a protective layer on the skin, hair or nails which can help protect the skin, hair or nails from physical, environmental or chemical injury. Specific protectants, such as moisturisers or sunscreens, optionally may be included in the cosmetic composition.

Brief Description of the Figures

Figure 1 is a graph illustrating the preferential crosslinking of involucrin to particulates in which Figure 1A is a comparison of crosslinking of cytosolic proteins of fibroblasts (solid circles) and keratinocytes (open triangles); and Figure 1B is a comparison of crosslinking of cytosolic proteins of keratinocytes (open triangles) and of involucrin (open circles).

Figure 2 is a graph illustrating the inability of other cytosolic proteins to compete with involucrin in crosslinking to particulates; keratinocyte particles were incubated with increasing amounts of unlabeled involucrin (circles) and unlabeled involucrin-free cytosol (triangles).

Figure 3 is a graph illustrating the detergent solubility of crosslinked involucrin attached to keratinocyte particles; keratinocyte particles were incubated under crosslinking conditions with increasing amounts of involucrin then boiled in sodium dodecyl sulfate (SDS). The soluble type II polymers are shown as solid circles and insoluble type III polymers as open circles.

15 Detailed Description

Proteins which are useful in the present composition and method comprise corneocyte proteins and polypeptide fragments thereof. Corneocyte proteins which are presently preferred include, for example, involucrin, loricrin, cornifin, trichohyalin, scieillin, profilaggrin and keratolinin. Involucrin has been described, for example, by Rice and Green (1979), *ibid.*; and Banks-Schlegel and Green (1981) *J. Cell Biol.*, 90:732-737. Loricrin has been described, for example, by Mehrel et al. (1990) *Cell*, 61:1103-1112; and Steven et al. (1990) *J. Struct. Biol.*, 104:150-162. Cornifin has been described, for example, by Marvin et al. (1992) *PNAS*, 89:11026-11030. Trichohyalin has been described, for example, by Fietz (1990) *J. Cell Biol.*, 110:427-436. Scieillin has been described, for example, by Kvedar et al. (1992) *Differentiation*, 49: 195-204. Profilaggrin has been described, for example, by Markova et al. (1993) *Mol. and Cell Biol.*, 13:613-625. Keratolinin has been described, for example, by Zettergren et al. (1982) *PNAS*, 81:238-242. Natural-sourced or recombinant proteins or polypeptides can be used.

The composition comprises an effective amount of one or more corneocyte proteins or polypeptides in a cosmetically acceptable vehicle. The composition is formulated to be suitable for topical application to mammalian skin, hair and/or nails. In a preferred embodiment, the composition further comprises a crosslinking agent. The crosslinking agent can be a chemical crosslinking agent, e.g., a difunctional or multifunctional compound having functional groups capable of forming ionic or covalent bonds with amino acids in the polypeptide chain. The crosslinking agent can also be an enzyme which is capable of inducing formation of bonds between molecules of the protein or polypeptide. Enzymes are preferred agents for this purpose. A particularly preferred enzyme is calcium-activated keratinocyte transglutaminase (hereinafter, "transglutaminase") which forms isopeptide bonds between glutamyl and lysyl residues of adjacent polypeptides. Transglutaminase preferably is added to the composition together with a concentration of calcium ions sufficient to render the enzyme enzymatically active. Corneocyte proteins applied to skin, hair or nails together with or following transglutaminase will be crosslinked to the outer layers of the skin, hair or nails, thereby forming the protective layer, or enhancing the protective properties of the stratum corneum.

In another preferred embodiment of the present invention, a composition comprising transglutaminase is applied to skin, hair or nails. The transglutaminase composition preferably contains a source of calcium ions to activate the enzyme. Alternatively, calcium ions may be applied subsequent to application of the transglutaminase composition. Application of transglutaminase and its activation by the calcium ions crosslinks corneocyte proteins which are present in the stratum corneum of the skin, hair or nails, thereby resulting in formation of the desired crosslinked layer.

Transglutaminase and the envelope precursor proteins can be extracted from natural sources; however, it is preferable to produce them by expression of the encoding cDNA's in suitable host cells. Various hosts have been used to produce recombinant proteins, e.g., bacterial cells, yeast, mammalian cells or insect cells infected with recombinant baculovirus. Genes encoding the corneocyte proteins are available. For example, complementary DNA (cDNA) encoding human involucrin (Eckert and Green, (1986) *Cell*, 46:583-589), human loricrin (Hohl et al. (1991) *J. Biol. Chem.*, 266:6626-6636), and human cornifin (Marvin et al., *ibid.*) have been isolated and sequenced. The cDNA encoding human keratinocyte transglutaminase has been described by Phillips et al. (1990) *PNAS, USA*, 87:9333-9337.

Baculovirus is the currently preferred host for producing the present proteins. Baculovirus seems to offer the highest levels of recombinant protein production together with correct processing of the protein, if necessary. Methods of producing recombinant proteins using baculoviruses are described, for example, by Piwnica-Worms in *Current Protocols in Molecular Biology*, Sections 16.8 through 16.11, John Wiley and

Sons, New York, N.Y. (1990). The most widely used baculovirus for expression of cDNA's is Autographa California, and is commercially available from Invitrogen Corp., San Diego, CA. In this system, the cDNA's are subcloned into the baculovirus transfer vector in the polyhedrin gene. SF9 or SF21 insect cells are cotransfected with the recombinant transfer vector and wild-type viral DNA. Five to ten percent of the transfer vector undergoes homologous recombination with the wild-type virus as shown by morphology of plaques. Recombinant plaques are isolated and the virus is grown on a large scale. Genes expressed under control of the viral polyhedrin promoter can account for more than half the total cell protein, and production rates of recombinant protein have been reported to range from 1 mg to 500 mg/l of culture (Piwnica-Worms (1990), *ibid*). The recombinant proteins produced by this method can be purified, for example, by gel filtration and ion exchange chromatography.

The proteins or polypeptides may be modified, if desired. Such modifications may include, for example, chemically derivatizing one or more amino acids or altering the amino acid sequence of the protein or peptide. These modifications can be used to impart selected qualities to the proteins or polypeptides, e.g., to render them more water-soluble, or more resistant to chemical, environmental or enzymatic attack. For example, selected amino acid functional groups can be derivatized and/or crosslinked, thereby increasing the resistance of the protein to degradation.

The amount of corneocyte proteins or polypeptides in the composition is the amount sufficient to provide a protective layer on the skin, hair or nails. The amount will vary depending on the formulation and the performance desired. In general, an amount of from about 0.1% to about 90% by weight protein or polypeptide will be sufficient for most purposes. Where the composition comprises only the crosslinking agent, the amount of crosslinking agent included in the composition will be an amount sufficient to induce formation of crosslinks between corneocyte proteins which are present in the stratum corneum. When transglutaminase is used, a source of calcium ions also must be applied either together with or subsequent to application of the enzyme.

The composition containing the proteins or polypeptides further comprises a cosmetically acceptable vehicle which is suitable for topical application to skin, hair and/or nails. For this purpose, the composition may comprise ingredients adapted to form a cream, gel, emulsion, liquid, suspension, nail coating, skin oil or lotion, for example. Ingredients which may be included in the composition include, for example, moisturizing agents, astringent agents, film-forming materials, surfactants, emollients, humectants, moisturizers, sunscreens, pigments or other proteins and/or fibers. Examples of such ingredients include various hydroxylated compounds, such as monomeric glycols, e.g., propylene glycol, ethyl alcohol, glycerin and butylene glycol, polymeric moisturizers such as polyglycerylmethacrylate, derivatives of palmitates and stearates, triglycerides of fatty acids, lanolin, vegetable or mineral oils, and waxes.

The nature of the vehicle will depend upon the method chosen for topical administration of the composition. The vehicle can itself be inert or it can possess physiological or pharmaceutical benefits of its own. The selection of a vehicle for this purpose presents a wide range of possibilities depending on the desired product form of the composition.

For purposes of the present invention, vehicles are substances which can act as diluents, dispersants, or solvents for the proteins or polypeptides which ensure that they can be applied to and distributed evenly over the skin, hair and/or nails at an appropriate concentration. The vehicle is preferably one which can aid formation of a protective layer on the skin. The vehicle or vehicles can comprise from 1 to 99.9999%, preferably from 50 to 99.5% and ideally from 90 to 99% by weight of the compositions.

Emollients which may be used in the composition include, for example, stearyl alcohol, glyceryl monoricinoleate, glyceryl monostearate, propane-1,2-diol, butane-1,3-diol, mink oil, cetyl alcohol, isopropyl isostearate, stearic acid, isobutyl palmitate, isocetyl stearate, oleyl alcohol, isopropyl laurate, hexyl laurate, decyl oleate, octadecan-2-ol, isocetyl alcohol, cetyl palmitate, dimethylpolysiloxane, di-n-butyl sebacate, isopropyl myristate, isopropyl palmitate, isopropyl stearate, butyl stearate, polyethylene glycol, triethylene glycol, lanolin, sesame oil, coconut oil, arrachis oil, castor oil, acetylated lanolin alcohols, petroleum, mineral oil, butyl myristate, isostearic acid, palmitic acid, isopropyl linoleate, lauryl lactate, myristyl lactate, decyl oleate, myristyl myristate.

Humectants which may be used include, for example, glycerin, sorbitol, sodium 2-pyrrolidone-5-carboxylate, soluble collagen, dibutylphthalate, gelatin.

Film-forming materials may include, for example, acrylic polymers, nitrocellulose polymers, ethyl acetate resins, butyl acetate resins, toluenesulfonamide resins, formaldehyde resins, polyvinylbutyral resins, polyester resins, and various other polymers and copolymers.

Pigments may include, for example, titanium dioxide, micas, iron oxides, barium lake, calcium lake, aluminum lake, bismuth oxychloride, zirconium lake and calcium oxides.

Other materials which can be used in the present compositions include solvents, thickeners and powders. Examples of each of these, are as follows:

Solvents may include water, ethyl alcohol, toluene, methylene chloride, isopropanol, n-butyl alcohol, castor oil, ethylene glycol monoethyl ether, diethylene glycol monobutyl ether, diethylene glycol monoethyl ether, dimethyl sulphoxide, dimethyl formamide and tetrahydrofuran.

Thickeners may include starch, gums such as gum arabic, kaolin or other clays, hydrated aluminum silicate, fumed silica, carboxyvinyl polymer, sodium carboxymethyl cellulose or other cellulose derivatives, ethylene glycol monostearate and sodium alginates.

Powders may include chalk, talc, fullers earth, colloidal silicon dioxide, sodium polyacrylate, tetra alkyl and/or trialkyl aryl ammonium smectites and chemically modified magnesium aluminum silicate.

The composition according to the invention optionally may comprise a perfume in an amount sufficient to make the composition acceptable to the consumer and pleasant to use. Usually, the perfume will form from about 0.01 to 10% by weight of the composition.

A protein stabilizing agent may be included in the composition. For example, skin contains natural proteases which might at least partially degrade the protein. Therefore, the presence of protein stabilizing agent such as a protease inhibitor can protect the protein. Examples of protein stabilizing agents include, for example, glycerol, ethylenediaminetetraacetic acid, cysteine, and proteinase inhibitors such as α_2 -macroglobulin, α_1 -antitrypsin, leupeptin, pepstatin, antipain, and cystatin. Cystatin is naturally present in epidermis and may be particularly useful for this purpose.

The present compositions preferably comprise one or more surfactants. The term "surfactants" refers to surface active agents which, when added to water, cause it to penetrate more easily into, or spread on the surface of another material, by reducing the surface tension of the water at the water-air or water-oil interface. By "surface active agent" is meant any compound that reduces surface tension when dissolved in water or water solutions.

The selection of a surfactant for this purpose presents a wide range of possibilities known in the art. Preferred examples of surface active agents include the following:

- (i) Anionic surfactants, such as metallic or alkanolamine salts of fatty acids for example sodium laurate and triethanolamine oleate;
 - alkyl benzene sulphones, for example triethanolamine dodecyl benzene sulphonate;
 - alkyl sulphates, for example sodium lauryl sulphate;
 - alkyl ether sulphates, for example sodium lauryl ether sulphate (2 to 8 EO);
 - sulphosuccinates, for example sodium dioctyl sulphonylsuccinate;
 - monoglyceride sulphates, for example sodium glyceryl monostearate monosulphate;
 - isethionates, for example sodium isethionate;
 - methyl taurides, for example Igepon T;
 - acylsarcosinates, for example sodium myristyl sarcosinate;
 - acyl peptides, for example Maypons and lamepons; acyl lactylates,
 - polyalkoxylated ether glycolates, for example trideceth-7 carboxylic acid;
 - phosphates, for example sodium dilauryl phosphate.
- (ii) Cationic surfactants, such as amine salts, for example sapamin hydrochloride;
 - quaternary ammonium salts, for example Quaternium 5, Quaternium 31 and Quaternium 18;
- (iii) Amphoteric surfactants, such as imidazol compounds, for example Miranol;
 - N-alkyl amino acids, such as sodium cocamino propionate and asparagine derivatives;
 - betaines, for example cocamidopropyle betaine
- (iv) Nonionic surfactants, such as fatty acid alkanolamides, for example oleic ethanolamide; esters or polyalcohols, for example Span; polyglycerol esters, for example that esterified with C₁₂-18 fatty acids and one or several OH groups;
 - Polyalkoxylated derivatives, for example polyoxy-polyoxyethylene stearate;
 - ethers, for example polyoxyethoxy lauryl ether;
 - ester ethers, for example Tween;
 - amine oxides, for example coconut and dodecyl dimethyl amine oxides. Mixtures of two or more of the above surfactants can be employed in the composition according to the invention.

The compositions of this invention may include a skin-structuring protein component. The term "skin-structuring protein" as used herein is intended to include natural proteins derived from animal or marine sources, as opposed to plant or vegetable sources, and which are substantive to the skin. Such animal proteins include, for example, high molecular weight glycoproteins, such as the fibronectins. Other useful animal proteins include soluble collagen, hydrolyzed animal collagen, keratins, striated muscle fiber or an extract thereof, hydrolyzed or partially hydrolyzed elastin, hydrolyzed silk, and soluble reticulin. The skin-

structuring protein component can comprise a mixture, as well as a single protein product.

The skin care compositions within the scope of this invention may include one or more astringent agents. Such agents include, for example, arnica flowers or extracts thereof, lower alkyl alcohols, witch hazel, boric acid, lactic acid, methol, camphor, zinc phenol sulphonate, aluminum acetate, aluminum sulfate, and zinc chloride or sulfate. For many purposes, a natural product, e.g., arnica extract, is preferred, especially in combination with a proteinaceous material, such as hydrolyzed marine protein and/or a vegetable protein as can be extracted from barley and other grains, for example.

The compositions of this invention may include a cellulosic film-former, if desired. Cellulosic film-formers include polysaccharides such as chitin, which can be obtained from marine sources. The chemical structure of the cellulosic film-former component is distinguished from that of the mucopolysaccharide component in predominantly containing monosaccharide repeating units, N-acetyl-D-glucosamine in the case of chitin.

If desired, the composition may contain UV-A and UV-B radiation filters, sunscreens, free-radical blockers, vitamin extracts, and antioxidants.

In addition to the elements described above, the skin care compositions of this invention may include cosmetically acceptable preservatives, antiseptics, pigments or colorants, fragrances, masking agents, and carriers, such as water and lower alkyl, e.g., C₁-C₄, alcohols, including ingredients to control the viscosity of the compositions.

Compositions in the form of hair dressing lotions may incorporate amionic, nonionic or cationic polymers in an aqueous, alcoholic or aqueous/alcoholic solution.

The quantities of the various ingredients in a composition within the scope of this invention can be varied over a wide range, it only being necessary that cosmetically effective amounts be present. In general, the primary moisturizing agent may comprise between about 0.25 and about 10 percent, desirably between about 0.3 and about 5 percent, by weight of the composition. The skin-structuring protein may comprise between about 0.05 and about 8 percent, desirably between about 0.05 and about 2.0 percent, by weight of the composition. The astringent agent, e.g., arnica combined with marine and vegetable protein, may comprise between about 0.1 and about 5 percent, preferably between about 0.1 and about 0.25 percent, by weight of the composition.

The present composition containing the proteins can be applied to the skin, hair and/or nails of humans in any of several different ways. For example, a composition containing the substrate proteins could be applied and, if crosslinking is desired, the enzyme (with calcium ions, if necessary) could be applied subsequently. Alternatively, the crosslinking agent may be added to the composition prior to its application. In another embodiment, the composition could be applied as an emulsion in which the enzyme (and the calcium ions) are held separate from the protein substrates in different phases and the two reagents are combined upon application to the skin or hair as the droplets containing each fuse and their contents become mixed. In yet another embodiment, a composition containing a crosslinker specific for corneocyte proteins is applied to the skin, hair or nails, thereby inducing crosslinking of corneocyte proteins already present in the stratum corneum of the skin, hair or nails. If transglutaminase is used, a source of calcium ions is applied together with or subsequent to application of the enzyme.

Since all components of the film should be substantially identical to their corresponding natural products, there should be no reason to remove the film. If, for any reason, removal should be desired, this could be accomplished by applying a solution of proteolytic enzymes. Among the enzymes that could be used are those occurring naturally in plants, such as papain (papaya), bromelin (pineapple), or ficin (ficus), or enzymes of microbial origin, such as subtilisin (bacterial) or pronase (fungal). These enzymes have broad specificity and some have been used clinically in the treatment of burns. Application in the form of a lotion or ointment would be limited to the shortest period necessary to hydrolyze the external film of cross-linked protein and possibly a few layers of corneocytes. The ability of pronase, bromelin or subtilisin to dissolve cross-linked envelopes has been demonstrated. Sun and Green, Cell, 9:511-521 (1978).

The present compositions and methods provide cosmetic compositions capable of forming a protective layer on skin, hair or nails which can mimic, or enhance the protective ability of the stratum corneum of natural skin. The use of corneocyte proteins which are naturally present in skin, hair and nails, particularly in a crosslinked state, permits a resistant layer to form which can protect skin and hair from environmental damage. The present corneocyte protein compositions may be applied as a cream or lotion, shampoo or hair gel, nail polish or coating or may be included in a foundation make-up formula, for example. Exemplary formulations for such cosmetic compositions are readily available, for example, in the Formulary section of the "happi" (household and personal products industry) journal, and other sources well known to those skilled in the art.

The invention is further illustrated by the following Exemplification.

EXEMPLIFICATION

In the following example, involucrin is extracted from human keratinocytes, purified, and tested for the ability to become crosslinked to a particulate fraction (from Simon and Green (1985) Cell,40:677-683).

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Involucrin and Other Cytosolic Proteins Compared as Substrates for Crosslinking to Keratinocyte Particulates

Cultured keratinocytes and fibroblasts were grown in the presence of [³⁵S]labeled methionine, and a crude extract containing cytosolic proteins was prepared from each cell type. The extract prepared from keratinocytes was passed through an immune affinity column to remove involucrin. After incubation of the labeled cytosolic proteins or of involucrin with unlabeled keratinocyte particulates under conditions permitting cross-linking, the particulates were centrifuged, washed twice with preparation buffer, and once with a solution of 2% SDS and 5% mercaptoethanol. The pellet was suspended in buffer and applied to the sample chamber of a gel electrophoresis apparatus. After electrophoresis to remove any residual uncross-linked proteins, the top of the gel was cut out and counted. The keratinocyte particulates were able to incorporate labeled cytosolic proteins of either cell type with about the same efficiency (Figure 1A). Within 1 hr, about 2% of the total labeled protein was cross-linked to the particulate fraction.

The labeled involucrin-free cytosol of keratinocytes was then compared with purified labeled involucrin for ability to become crosslinked to keratinocyte particulates (Figure 1B). The involucrin was initially 4-5 times more effective, and 10% became crosslinked into aggregates within 80 min. Fibroblast particulates and mixtures of fibroblast particulates and cytosol were incapable of crosslinking involucrin into SDS-insoluble polymers.

While involucrin was preferred to other cytosolic proteins in the crosslinking to particulates, the preference factor was smaller than that observed for putrescine labeling. It was therefore of interest to determine whether unlabeled cytosolic proteins were able to compete with involucrin in the cross-linking. Keratinocyte particulates were incubated with [³⁵S]involucrin and with unlabeled involucrin or involucrin-free cytosolic protein. As seen in Figure 2, unlabeled involucrin effectively competed with labeled involucrin, reducing the formation of labeled product by over 50%. A higher concentration of involucrin-free cytosol exerted no significant reduction in cross-linking of labeled involucrin.

Formation of Smaller Aggregates Containing Polymerized Involucrin

The cross-linking of involucrin into large aggregates may take place in stages. When keratinocyte particulates were incubated with [³⁵S]involucrin for 30 min, they were found to form involucrin-containing polymers of different sizes. Some of these polymers remained soluble and were separated from the particulates by centrifugation for 15 min at 15,000 x g. When subjected to electrophoresis, the supernatant containing these polymers gave bands corresponding to approximate molecular weights of 200 kd and 300 kd. The smaller polymer was generally more abundant. These proteins (Type 1 polymers) may be homopolymers of involucrin.

In the centrifuged particulates, there were two other kinds of polymers. When extracted with a solution containing 2% SDS and 5% mercaptoethanol at 100°C and recentrifuged, Type II polymers became soluble, and Type III polymers remained large enough to be sedimentable. Type II polymers had molecular weights greater than 400 kd, as they did not enter a 5% acrylamide gel even after electrophoresis for 4 hr. The proportion of crosslinked involucrin in polymers of Types II and III depended on the concentration of involucrin. At low concentrations, 30-50% of labeled involucrin polymers were of Type II (Figure 3). At higher concentrations of labeled involucrin, nearly all polymers were of Type III. This suggests that the Type II polymers may be precursors of the Type III.

50 The Role of Involucrin in the CrossLinking of Particulates

When transglutaminase is activated in intact keratinocytes by the introduction of Ca⁺⁺ not only involucrin but also proteins resident in the cell membrane become cross-linked (Simon and Green (1984) Cell,36:827-834). In order to test for a role of involucrin in the crosslinking of these proteins, the effect of involucrin on the crosslinking *in vitro* of proteins of the particulate fraction was examined.

Cultured keratinocytes were grown in the presence of [³⁵S]labeled methionine, and the labeled particulate fraction was isolated. This fraction was then incubated under crosslinking conditions, and the incorporation of label into SDS-insoluble cross-linked material was measured (Table 1). An appreciable

background of crosslinked material was present prior to incubation; this crosslinking took place during cultivation, when some crosslinked envelopes form spontaneously, the number increasing with the age of the culture. During incubation of the particulate fraction under crosslinking conditions, more labeled protein became crosslinked. Addition of involucrin to the labeled particulates produced a further increase in the amount of labeled protein crosslinked. The addition of involucrin-free cytosolic protein at the same concentration (or up to 25-fold higher concentration) had no such effect. This shows that while soluble proteins other than involucrin may crosslink to proteins of the particulate fraction, the average cytosolic protein does not share the specific ability of involucrin to promote the crosslinking of particulate proteins.

10 Discussion

As shown earlier, the crosslinked envelope of the keratinocyte appears to be assembled from a mixture of membrane bound and cytosolic constituents (Rice and Green (1979) Cell,18:681-694; Simon and Green (1984) ibid). Because of the difficulty in obtaining a pure membrane fraction from keratinocytes, the assays used in this study employed crude particulates. However, proteins present in the particulates and subject to cross-linking have been shown to be membrane associated (Simon and Green (1985) ibid).

As demonstrated here, the particulate fraction of keratinocytes, but not a similar fraction prepared from fibroblasts, is able to crosslink cytosolic protein to make aggregates *in vitro*. This difference between the two cell types is correlated with the fact that the transglutaminase of keratinocytes is predominantly linked to particulates (Thacher and Rice (1983) Fed. Proc., 42(7):1925 Abstr. 980), whereas that of fibroblasts is predominantly cytosolic. The correlation is rendered still more significant by the finding that keratinocytes possess a transglutaminase different from that of fibroblasts or other cell types (Phillips et al. (1990) PNAS, 87:9333-9337). It IS assumed that any cytosolic protein rendered insoluble in SDS and unable to enter polyacrylamide gels is crosslinked to proteins within the particulate fraction. As shown earlier, membrane proteins of 195 kd and 210 kd became crosslinked in intact cells rendered permeable to Ca⁺⁺ (Simon and Green (1984) ibid); over 50% of each of these proteins also became SDS-insoluble in the reconstituted system described here.

Transglutaminase is able to couple putrescine and other amines to monomeric protein (Clark et al. (1950) Arch. Biochem. Phys.,79:338-354; Folk and Chung (1973) Adv. Enzymol.,38:109-191). Indeed, it was by its ability to act as an amine acceptor in the presence of cytosolic enzyme that involucrin was first identified (Rice and Green (1979) ibid). The present study shows that the particulate fraction of keratinocytes is much more effective than the cytosol in catalyzing amine labeling of involucrin. In the reaction with putrescine, the particulate transglutaminase preferred, as a substrate, involucrin to the average cytosolic protein by a factor of at least 80-fold. This is probably an underestimate, since some of the putrescine-labeled involucrin was probably crosslinked into aggregates and escaped detection. It was known from amino acid analysis of involucrin that 45% of all residues consisted of glutamic acid or glutamine (Rice and Green (1979) ibid). Other studies have shown that in at least part of the involucrin molecule, glutamine alone accounts for about 25% of the residues. Nearly 15% of involucrin is composed of leucine residues, known to be important neighbors of crosslinking glutamine residues in casein and model substrates (Gorman and Folk (1984) J. Biol. Chem.,259:9007-9010). It is perhaps for these reasons that involucrin is a better transglutaminase substrate than most other proteins.

When keratinocyte particulates were tested for ability to discriminate between involucrin and other cytosolic proteins in crosslinking to form large SDS-insoluble aggregates, they preferred involucrin to other proteins, but by a factor of only 5 or less. There are several possible explanations for the relatively low figure. One is that *in vitro*, the involucrin may not be properly located with respect to particulate proteins to which it must crosslink, and the true selectivity of the system is not expressed. A second possibility is that involucrin is subject to proteolysis *in vitro*, particularly in the presence of Ca⁺⁺ and free SH groups, both of which are required for transglutaminase activity. After the incubation under cross-linking conditions, residual soluble involucrin frequently gives a diffuse band in gel electrophoresis. Intactness of the molecule may be more important for crosslinking of the protein to particulates than for its ability to act as putrescine acceptor. Finally, although its ability to couple to putrescine shows that involucrin acts as an amine acceptor in forming the isopeptide bond, other cytosolic proteins may act as amine donors. Consistent with this possibility is the fact that in cross-linking to particulates, other cytosolic proteins are ineffective as competitors against labeled involucrin (Figure 2).

The total concentration of all cytosolic proteins in the keratinocyte is about 25 times higher than that of involucrin. If the preference factor for involucrin in cellular crosslinking were only 5-fold, it might be expected that involucrin would account for only about 20% of the cytosolic contribution to the crosslinked envelope. However the crosslinking of cytosolic proteins by keratinocyte particulates *in vitro* is saturated at

a protein concentration of about 5 mg/ml, (much lower than the cellular cytosolic protein concentration of about 65 mg/ml). Even though a maximal rate of crosslinking was not reached at the highest involucrin concentration tested in vitro (0.6 mg/ml), the amount of involucrin crosslinked was equal to the saturating value for all other cytosolic proteins. If the proportion of involucrin and other cytosolic proteins crosslinked in vitro reflects their behavior in the intact cell, the contribution of involucrin to the envelope would not be less than 50% of the total cytosolic contribution.

In the keratinocyte, the crosslinking system has evolved considerably to form the substantial envelope that underlies the plasma membrane in the final stage of terminal differentiation. The cytosolic protein involucrin not only contributes to the mass of the envelope but also promotes the polymerization of other proteins likely to be associated with the plasma membrane. Other cytosolic proteins are not able to substitute for involucrin in this function (Table 1). In this way, involucrin may also aid in securing the envelope to the plasma membrane.

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Table 1
Crosslinking of Proteins of the Particulate Fraction In Vitro and the Effect of Involutrin % of Total [35 s] in Form of Polymer after Incubation In Vitro

Experiment #	Background*	No Added Protein		+ Involutrin-free cytosolic protein (2.0 μ g)		+ Involutrin 2.0 μ g
1	4.4	7.2		7.0		11.8
2	2.0	9.3		9.2		12.5
3	0.4	8.8		8.7		11.0
4	0.8	10.4		10.5		12.9
5	1.9	7.4		7.4		10.7

*Polymerized in intact cells prior to incubation.

A particulate fraction, prepared from labeled keratinocytes and containing 50 μ g of protein, was incubated for 30 min under cross-linking conditions. After the reaction was terminated, the particulates were washed, first with preparation buffer, and then with a solution of SDS containing mercaptoethanol. Any remaining unpolymerized material was removed by electrophoresis, and the polymers remaining in the stacking gel were counted. Controls incubated in the presence of either EDTA or cystamine showed no increase over background.

Methods and Materials

5 Cell Culture

Human epidermal keratinocytes (strain N) were grown in the presence of lethally irradiated 3T3 cells (Rheinwald and Green (1975) *Cell*, **6**: 317-330) using a 3:1 mixture of the Dulbecco-Vogt modification of Eagle's medium and Ham's F-12 medium (Ham (1965) *Proc. Natl. Acad. Sci. USA*, **53**: 288-293).
 10 Supplements were as follows: fetal calf serum, 10%; hydrocortisone, 0.4 µg/ml; insulin, 5 µg/ml; transferrin, 5 µg/ml; triiodothyronine, 2×10^{-9} M; cholera toxin, 10^{-10} M; and adenine, 1.8×10^{-4} M (Peehl and Ham (1980) *In Vitro*, **16**: 526-538; Wu et al. (1982) *Cell*, **31**: 693-703. Beginning 3 days after inoculation, epidermal growth factor (EGF) prepared according to Savage and Cohen (1972) (*J. Biol. Chem.*, **247**: 7609-7611) was added to the medium to 10 ng/ml (Rheinwald and Green (1977) (*Nature*, **265**: 421-424). Human
 15 fibroblasts and 3T3 cells were grown in Dulbecco-Vogt modification of Eagle's medium supplemented with 10% fetal calf serum or 10% calf serum, respectively. All cultures were incubated at 37°C in an atmosphere containing 10% CO₂, and the medium was changed twice weekly.

Preparation of Particulates and Cytosol

20 Keratinocytes or fibroblasts grown to confluence were washed twice with ice cold 0.6 mM HEPES, pH 7.4; 0.15 M NaCl; 0.4 mg/ml bovine serum albumin; 0.2 mM phenylmethyl sulfonyl fluoride (PMSF); 10 mM ξ -amino caproic acid; 10 mM phenylmethyl sulfonyl fluoride (PMSF); 10 mM EDTA; and 1 mM MgCl₂. The suspension was then sonically disintegrated with 5 bursts at a setting of 6 on a Branson Sonifier, and the
 25 particulates and cytosol were separated by centrifugation for 30 min at 1000,000 x g. The fibroblast and keratinocyte particulates were then washed twice with the preparation buffer, centrifuging for 15 min at 30,000 x g and 15,000 x g, respectively.

Protein Purification and Antiserum Production

30 Involucrin was purified either according to the procedure of Rice and Green (1979) *ibid*, or Etoh et al. (1986) *Biochem. Biophys. Res. Comm.*, **136**: 51-56. Protein concentration was measured by the procedure of Lowry et al. (1951), *J. Biol. Chem.*, **193**: 265-275). Batches of involucrin prepared by the two methods were indistinguishable.

35 To prepare antiserum 100 µg of involucrin was dissolved in isotonic buffer containing 50% Freund's complete adjuvant and injected intradermally into rabbits. Booster injections, containing 50 µg of involucrin in 50% Freud's incomplete adjuvant, were given every 4 weeks thereafter. Animals were bled from the ear 1 week after each booster injection.

40 Preparation of Involucrin-Free Cytosol

Involucrin-free cytosol was obtained by affinity chromatography using a column of anti-involucrin antibody coupled to Sepharose 4B (Pharmacia). An immunoglobulin fraction was first prepared by precipitating the involucrin antiserum with ammonium sulfate at 40% saturation. The precipitated immunoglobulin fraction
 45 was dialyzed 3 times against 1 liter of isotonic phosphate buffer. The antibodies specific for involucrin were then obtained by affinity chromatography using a column of Sepharose 4B coupled to involucrin. The column was prepared using 300 µg of involucrin dissolved in 1.5 ml of 0.1 M sodium bicarbonate and dialyzed against a solution containing 0.1 M sodium bicarbonate and 0.5 M sodium chloride. The involucrin solution was then added to 0.3 g of CNBr-activated Sepharose, and the coupling was carried out according
 50 to the manufacturer's recommendations. 1.0 ml of the immunoglobulin fraction obtained from involucrin antiserum was added in a 1.0 ml volume to a column of involucrin-Sepharose. The column was then washed with 6 volumes of 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. No anti-involucrin antibody could be detected in this wash. The specifically bound antibody was then eluted with 0.1 M glycine-HCl, pH 2.5. Immunoblot analysis of the eluate showed that 30% of the antibody activity was recovered. To prepare the
 55 anti-involucrin antibody column, the eluted antibody (213 µg) was neutralized with 1 M NaOH within 10 min and coupled to Sepharose as described above. Coupling was 67% effective.

To separate involucrin from other cytosolic proteins, unfractionated cytosol was added in a 0.5 ml volume to the column containing anti-involucrin antibody and the column was washed with preparation

buffer. As determined by immunoblot analysis, this column was capable of removing greater than 99.98% of the involucrin contained in 1 mg of cytosolic protein. The involucrin was eluted from the column with 0.1 M glycine-HCl, pH 2.5, and was neutralized. Electrophoretic analysis of the eluate showed that the protein present in the involucrin band accounted for over 95% of the total.

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CrossLinking Assays

All experiments were carried out at 37°C in a reaction volume of 50 µl containing preparation buffer. Crosslinking was activated by the addition of CaCl₂ to a final concentration of 10 mM. Controls contained either 20 mM EDTA and no CaCl₂, or 20 mM cystamine.

Two types of assay were employed. The first measured the covalent crosslinking of [³H]putrescine to protein (Folk and Cole, (1966) *Biochim. Biophys. Acta*, **122**: 244-264). No exogenous substrate (such as the commonly employed casein) was added; the acceptor protein was in all cases involucrin or other cellular proteins. In some experiments, incorporation of the putrescine into total trichloroacetic acid-precipitable material was determined. The precipitate was washed 4 times with 10% ice cold trichloroacetic acid, dissolved with sodium hydroxide, and reacidified with hydrochloric acid prior to counting. This assay measured both incorporation into aggregates, as well as soluble proteins. To measure incorporation into soluble proteins alone (either purified involucrin or a mixture of cytosolic proteins), the reaction products were electrophoresed, and the gels were fixed and washed in 10% acetic acid-methanol overnight. The gels were then either treated with Enhance (New England Nuclear), dried and fluorographed on X-OMat AR film at -70°C to show labeling in discrete protein bands, or each separation lane was excised, incubated overnight at 37°C in 3% Protosol in Econofluor (New England Nuclear), and counted.

The second crosslinking assay measured the formation of polymeric aggregates from labeled involucrin, labeled cytosolic proteins, or labeled particulate proteins. Following the reaction, the samples were centrifuged for 15 min and the pellet was washed twice in preparation buffer containing bovine serum albumin (BSA) and once with 2% SDS and 5% mercaptoethanol at 100°C. After the final centrifugation, the pellet was suspended in Laemmli sample buffer and any residual monomeric protein was removed by electrophoresis through a 5% polyacrylamide gel for about 4 hr at 30 mA. The stacking gel and the very top of the separating gel (1 mm) were cut out, treated with Protosol, and counted. As expected for a transglutaminase-catalyzed reaction (Clark et al. (1959) *ibid*; Folk and Chung (1973) *ibid*; Lorand et al. (1978) *J. Supramol. Struct.*, **9**:427-440), crosslinking depended on the presence of calcium ions and was inhibited by cystamine, histamine, ethanolamine, or putrescine. The apparent K_M of calcium, for each of these crosslinking reactions, was 0.1-0.2 mM.

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Equivalents

Other embodiments of the present invention in addition to those specifically disclosed herein and which are equivalent thereto will be readily apparent to those skilled in the art. Such equivalents are intended to be encompassed within the scope of the following claims.

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Claims

1. A cosmetic composition suitable for topical application to mammalian skin, hair or nails comprising at least one corneocyte protein or polypeptide in a cosmetically acceptable vehicle.
2. The composition of claim 1 wherein the corneocyte protein or polypeptide selected from the group consisting of involucrin, loricrin, cornifin, trichohyalin, scellin and profilaggrin.
3. The composition of claim 1 wherein the protein or polypeptide is produced by recombinant methods.
4. The composition of claim 3 wherein the amino acid sequence of the protein or polypeptide has been modified and differs from the amino acid sequence of the natural-sourced protein or polypeptide.
5. The composition of claim 1 wherein the protein or polypeptide has been modified by chemically derivatizing at least one amino acid of the protein or polypeptide.
6. The composition of claim 1 further comprising a crosslinker which induces formation of crosslinks between different protein or polypeptide molecules.

7. The composition of claim 6 wherein the crosslinker comprises an enzyme.
8. The composition of claim 7 wherein the enzyme is transglutaminase.
- 5 9. The composition of claim 8 further comprising calcium ions.
10. The composition of claim 1 comprising an emulsion having a continuous phase and a discontinuous phase wherein the protein or polypeptide is contained within one of the phases.
- 10 11. The composition of claim 10 wherein the emulsion further comprises a crosslinker which induces formation of covalent bonds between different protein or polypeptide molecules.
12. The composition of claim 11 wherein the crosslinker is contained in the phase not occupied by the protein or polypeptide.
- 15 13. The composition of claim 1 wherein said cosmetically acceptable vehicle comprises ingredients forming a cream, gel, emulsion, skin oil, nail coating or lotion.
14. A composition suitable for topical application to mammalian skin, hair or nails comprising a crosslinking agent capable of forming crosslinks between externally exposed proteins or polypeptides present in the stratum corneum of said skin, hair or nails in a cosmetically acceptable vehicle.
- 20 15. The composition of claim 14 wherein said crosslinking agent comprises an external crosslinker which forms a crosslink between amino acid residues of the proteins or polypeptides.
- 25 16. The composition of claim 15 wherein the crosslinking agent comprises an enzyme which induces formation of covalent bonds between molecules of the protein or polypeptide.
17. The composition of claim 16 wherein the enzyme is transglutaminase.
- 30 18. The composition of claim 17 further comprising calcium ions.
19. A method for providing a protective layer on mammalian skin, hair or nails comprising applying to the skin, hair or nails a cosmetic composition according to anyone of claims 1 to 13 suitable for topical application comprising a cosmetically acceptable vehicle and an amount of a corneocyte protein or polypeptide sufficient to form said protective layer.
- 35 20. A method for providing a protective layer on mammalian skin, hair or nails comprising applying to the skin, hair or nails a cosmetic composition according to anyone of claims 14 to 18 suitable for topical application comprising a cosmetically acceptable vehicle and an amount of a crosslinking agent which is capable of inducing formation of crosslinks between molecules of the protein or polypeptide thereby forming said protective layer.
- 40 21. The method of claim 20 wherein said cosmetically acceptable vehicle comprises ingredients forming a cream, gel, emulsion, skin oil, nail coating or lotion.
22. A modified corneocyte protein or polypeptide.
- 45 23. The modified protein or polypeptide of claim 22 wherein the corneocyte protein or polypeptide is selected from the group consisting of involucrin, loricrin, cornifin, trichohyalin, scieillin and profilaggrin.
24. The modified corneocyte protein or polypeptide of claim 22 wherein the modification comprises chemically derivatizing at least one amino acid of the protein or polypeptide.
- 50 25. The modified protein or polypeptide of claim 22 which is produced by recombinant methods.
26. The modified protein or polypeptide of claim 25 wherein the modified protein or polypeptide has an amino acid sequence which differs from the amino acid sequence of the natural-sourced protein or

polypeptide.

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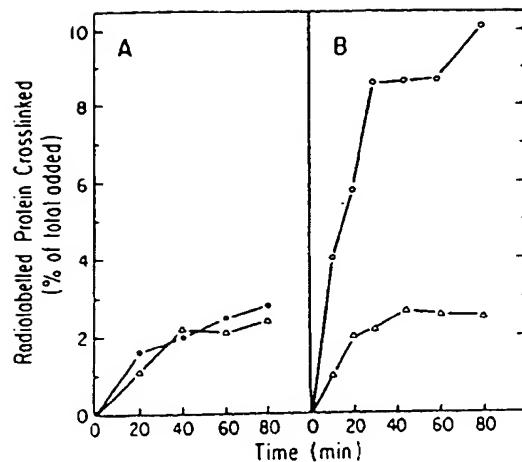


Figure 1

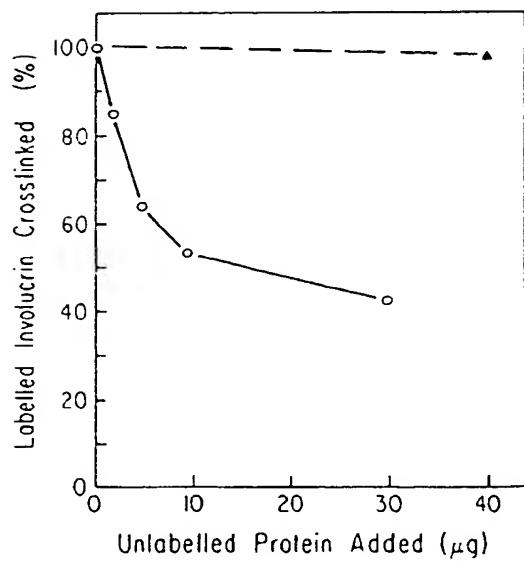


Figure 2

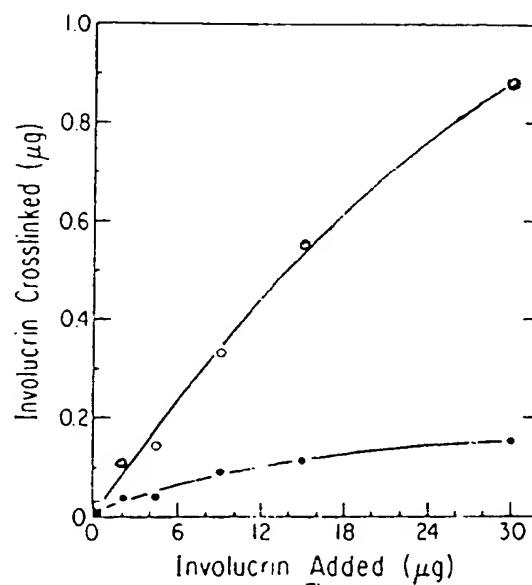


Figure 3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 93 40 2240

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.)
X	EP-A-0 285 474 (C.I.R.D.) * page 8, line 58 - line 62; claim 1 * ---	1,2	A61K7/48 C07K15/06
X	EP-A-0 511 116 (CLONATEC S.A.) * column 3, line 8 - line 46; claims 1-24; example 2 * ---	22,23,26	
X	COSMETICS & TOILETRIES vol. 103, no. 2, February 1988 pages 89 - 91 94 G. RIALDI ET AL. 'Filaggrin Overview. Functions and Cosmetic Aim.' * the whole document * ---	22,23,26	
T	CHEMICAL ABSTRACTS, vol. 120, no. 17, 25 April 1994, Columbus, Ohio, US; abstract no. 210039, U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES 'Cloning of DNA encoding mammalian trichohyalin and ...' * abstract * & US-A-56 200 (P. STEINERT) 15 September 1993 -----	1-26	
TECHNICAL FIELDS SEARCHED (Int.Cl.)			
A61K C07K			
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
THE HAGUE	14 July 1994	Willekens, G	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
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